NITROGEN SOURCE: AN EFFECTIVE COMPONENT FOR THE GROWTH, AND VIABILITY OF *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS*  

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**MATERIALS AND METHODS**

**Lactobacilli MRS Agar Medium**

MRS agar medium was prepared by completely dissolving 55g of MRS (Neogen Co, Michigan, USA) and 0.5g of L-Cysteine in 1 L of deionized water (DW). Difco agar powder (15g) was added, and the agar medium was sterilized at 121οC for 15 min and then cooled in a water bath. All freshly-prepared media in the study were poured into sterile petri dishes and stored at 4 ºC until further needed.

**Modified Reinforced Clostridial Medium-Pyruvate (mRCM-PYR)**

A reinforced clostridial medium according to Oyeniran *et al.* (2020) was optimized for selectivity and accurate enumeration of *Lb. bulgaricus* by dissolving 10g peptone #3, 10g beef extract, 5g yeast extract, 5g sodium chloride, 3g sodium acetate, 2g K2HPO4, 0.1g uracil, 0.25g calcium chloride, 5g dextrose, 5g fructose, 10g maltose, 2g sodium pyruvate, 0.2% Tween 80 and 0.5g L-Cysteine in 1 L DW. The final pH (6.0 ± 0.2) of the solution was obtained using 6N HCl before the addition of 0.008% aniline blue and 15g agar. The medium was autoclaved at 121οC for 15 min, cooled in a water bath, and poured into sterile petri dishes. All freshly-prepared media in sterile petri dishes were stored at 4ºC until needed.

**Validation of Nitrogen Sources as Growth Medium**

Five yeast samples (X-Seed Nucleo Max, X-Seed KAT, X-Seed Peptone, X-Seed Carbo-Peptone, and X-Seed Carbo-Max) provided by Ohly GmbH, Germany were used and tested in different blends and proportions during the preliminary study. X-Seed Carbo-Peptone, and X-Seed Carbo-Max were products under development by Ohly GmbH. The preliminary work thus confirmed X-
Seed Nucleo Max, X-Seed KAT, and X-Seed Carbo-Max yeast samples as superior alternative complex nitrogen sources for use in the study. The Supplementary Table S2 highlights the total percentage of protein and the total percentage of nitrogen in X-Seed Nucleo Max, X-Seed KAT, and X-Seed Carbo Max, respectively.

**Optimized Lactobacilli Growth Medium**

The growth medium was prepared similarly to the composition of standard deMan Rogosa Sharpe (MRS) medium as follows: completely dissolving 20g dextrose, 3g potassium phosphate dibasic, 3g ammonium citrate, 0.2g magnesium sulphate, 3g sodium acetate, 3g sodium pyruvate, 0.1g manganese sulphate, 0.2% Tween 80, 0.1g Uracil, 0.35g calcium chloride, 0.5g L-Cysteine, 2g X-Seed Nucleo Max, optimized proportions of blends of X-Seed KAT, and X-Seed Carbo Max (5g and 10g) in 1 L of deionized water (DW). This medium was adjusted to a final pH of 6.4 ± 0.2, using 6N HCl. Ten ml of the resultant solution was then dispensed into test tubes, autoclaved at 121°C for 15 min, and then cooled at room temperature.

**Culture Propagation in Growth Medium with Different Nitrogen Blends**

After 16 hours of growth in MRS and reaching an optical density (OD_{610nm}) between 0.7 and 0.9, the strains (S9 and LB6) were individually centrifuged at 5000 rpm for 20 minutes at 4°C (Thermo Scientific Sorvall ST 16R). The resultant supernatant was decanted, and cells were washed in a 0.1M phosphate-buffered saline (PBS) solution. The washed cells were then serially diluted ten-fold in peptone water. From the third-fold dilution, 250µl was inoculated in duplicates into each batch of test tubes as follows: MRS broth (control), basal growth medium with a nitrogen source (2g/L of X-Seed Nucleo Max: N-Max), basal medium with 2g/L of Nucleo Max supplemented with 5g/L and 10g/L Carbo Max respectively (C-Max 5% and C-Max 10%), basal medium with 2g/L of Nucleo Max supplemented with 5g/L and 10g/L KAT respectively (KAT 5% and KAT 10%), basal medium with 2g/L of Nucleo Max supplemented with 5g/L Carbo Max and 5g/L KAT respectively (KCMax 5/5), basal medium with 2g/L Carbo Max and 10g/L KAT respectively (KCMax 5/10), basal medium with 2g/L of Nucleo Max supplemented with 10g/L Carbo Max and 5g/L KAT respectively (KCMax 10/5), and basal
medium with 2g/L of Nucleo Max supplemented with 10g/L Carbo Max and 10g/L KAT respectively (KCMax 10/10). The complex nitrogen source blends used in the present study are shown in Supplementary Table S3. All test tubes with the different batches of nitrogen sources were vortexed and anaerobically incubated at 42°C for 12 hours, and the growth of cultures was measured at time intervals of 0, 6, and 12 hours at OD_{610nm}. The initial bacterial population was determined using the bacterial enumeration method.

**Determination of pH values for the different growth media**

The pH values of each test tube sample were measured in duplicates at the start (0 h) and end (12 h) of the fermentation period using a pH meter (Accumet AB 150, Fisher Scientific, Pittsburgh, PA). The pH readings were taken after calibration with standard pH buffers 4.0 and 7.0. The pH electrode was rinsed thoroughly with deionized water between the different samples.

**Determination of buffering capacity**

The initial pH values of all the nitrogen-supplemented media; N-Max, C-Max 5%, C-Max 10%, KAT 5%, KAT 10%, KCMax (5/5), KCMax (5/10), KCMax (10/5), KCMax (10/10), and MRS (control) were stabilized and adjusted to pH 6.5. A standard 0.1N of hydrochloric acid (HCl) was prepared for sample titration. Two aliquots (50ml) of each sample were placed in a 100ml beaker, and 1 mL of HCl acid was slowly titrated into each sample in order to reach a pH of 4.5.

**Bacterial Enumeration**

The bacterial growth of the two strains (LB6, and S9) was monitored via spectrophotometric measurement of the strains’ optical densities (OD_{610nm}) at different time intervals (0, 6, and 12 hours) using a Thermo Fisher Scientific Evolution 201 UV-visible spectrophotometer (Thermo Fisher Scientific, Inc. Waltham, MA, USA). The uncultured media were used as blanks during the spectrophotometric measurement, and the initial and final bacterial populations were determined. At the end of the fermentation period (12 h), a 1 mL sample was drawn from each inoculated sample and serially diluted in 0.9% peptone water. Appropriate dilutions were then surface plated (100 µl) onto duplicate MRS and mRCM-PYR agar plates. All plates were anaerobically incubated for 48 h at 42°C, and plates with 25 – 250 colonies were counted. The bacterial populations were then expressed in Log CFU/mL.
Determination of buffering capacity with L-Arginine and L-Histidine supplementation

The different nitrogen supplemented media were then prepared with and without the addition of 4g/L of L-Arginine and L-Histidine respectively and adjusted to pH 6.5. A standard 0.1N of hydrochloric acid (HCl) was prepared for sample titration. Two aliquots (50ml) of each sample were placed in a 100ml beaker, and 1 mL of 0.1N HCl acid was slowly titrated into each sample. The pH reading was recorded for each until the pH of the solution reached 4.5. The buffering capacity was then determined by adding the total amount of HCL acid consumed in order to reach the endpoint of pH 4.5.

Bacterial enumeration in growth media supplemented with and without L-Histidine

Bacterial growth and population counts were determined in the growth media (KCMax (5/10), KCMax (10/5), and KCMax (10/10) with and without L-histidine supplementation. A high and a low concentration (4g/L and 0.5g/L) of L-histidine were supplemented with the selected media. The control samples were MRS, and the standard growth media of KCMax (5/10), KCMax (10/5), and KCMax (10/10) had no L-histidine supplementation. The S9 strain of Lb. bulgaricus was used for this study and anaerobically fermented in the various growth media at 42°C for 12 hours. At the end of the fermentation period (12 h), a 1 mL sample was drawn from each inoculated sample and serially diluted in 0.9% peptone water. Appropriate dilutions were then surface plated (100 µl) onto duplicate MRS and mRCM-PYR agar plates. All plates were anaerobically incubated for 48 h at 42°C and plates with 25 – 250 colonies were counted. The bacterial populations were then expressed in Log CFU/mL.

Impact of calcium on the growth of Lb. bulgaricus in modified growth medium

Fifty (50) ml duplicate samples of the modified growth medium KCMax (10/10) were prepared with each containing varying concentrations of calcium; 4g/L (0.4%), 8g/L (0.8%) and 12g/L (1.2%), respectively. The control samples were prepared similarly but without calcium supplementation. The different calcium supplemented media and the control media were then inoculated with 1ml each of the S9 Lb. bulgaricus strain and were anaerobically fermented at 42°C for 12 hours. The pH measurements were taken, and population counts were determined by the enumeration method at the end of the fermentation period. Bacterial cells were then harvested by
centrifugation at 5000rpm for 15 minutes at 4°C. One set was washed with 50ml sterile deionized water, centrifuged a second time, suspended in sterile deionized water, and vortexed in order to obtain a uniform mixture. The other set was treated similarly but without washing with deionized water. Both samples were sonicated with a Branson Sonifier 250 unit for 4 minutes in order to disrupt the cells and the samples were rapidly cooled with ice. Samples were then filter sterilized through a filter with a pore size of 0.2µm in order to obtain a clear filtrate. Calcium content was finally determined in the clear filtrates by the Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) method using an Optima 800 ICP-OES unit (Perkin Elmer Company, MA, USA).

**Impact of calcium on the freeze-stability and viability of *Lb. bulgaricus***

Cells from the S9 strain of *Lb. bulgaricus* were harvested from overnight cultures, washed with phosphate-buffered saline (PBS) solution, serially diluted and appropriate dilutions were then inoculated into 50ml of MRS media and KCMax (10/10) respectively. All samples were performed in triplicates and anaerobically incubated at 42°C for 12 hours. The samples were then centrifuged at 5000 rpm for 15 minutes at 4°C following the fermentation period, in order to harvest the cell pellets. Bacterial cells from both the MRS and KCMax (10/10) media were then washed with and without 0.4% calcium chloride solution. Cells washed without the calcium chloride solution were assigned as the control and were only washed with sterile deionized water. Bacterial cells from both treatments were thinly spread in petri-dishes and pre-frozen overnight at -80°C before freeze-drying. Pre-frozen cells were freeze-dried in a Labconco unit for 48 hours. The freeze-dried samples were resuspended into 3ml of skimmed milk supplemented with 0.1ml yeast extract and 10µl Tween 80 in order to enhance the recovery of stressed bacterial cells. The mix was then incubated at 42°C for 6 hours, serially diluted with peptone solution, and 100µl of appropriate dilutions were subsequently plated in duplicates by the enumeration method. Plates were anaerobically incubated at 42°C for 48 hours, and colonies were counted.
RESULTS AND DISCUSSION

**Preliminary Study (validation of the nitrogen source on the growth of Lb. bulgaricus strains)**

The different nitrogen sources, X-Seed Nucleo Max (N-Max), X-Seed KAT (KAT), X-Seed Peptone (Pep), X-Seed Carbo Peptone (C-Pep), and X-Seed Carbo Max (C-Max) were validated and tested in different blends and formulations (Supplementary Table S3). Consequently, X-Seed Nucleo Max, X-Seed KAT, and X-Seed Carbo Max were considered as superior complex nitrogen sources and were blended as the optimized growth media. The viability of all the strains is shown in Supplementary Figure S1 and is linked to the first formulated media (Supplementary Table S3). Formulations two, and four did not yield promising growth results; however, formulation three (Supplementary Figure S2) included a basal medium with 2g/L of Nucleo-Max (N-Max) in conjunction with various blends of KAT (K) and Carbo-Max (C-Max) yielded exceptional results.

Several trials were done with various strains of Lb. bulgaricus until a final optimized growth media formulation with comparable results to MRS was obtained. The first formulation had a blend of 1% (w/v) of the different nitrogen sources, and five (5) strains (E22, S9, LB6, RDA, and ATCC 11842) were used. MRS was also used as a control medium in all of the preliminary formulations. Bacterial growth was monitored for eight (8) hours at an incubation temperature of 42°C. Although the optical density (OD$_{610nm}$) results of the strains in the first formulated growth media were not promising, MRS (the standard control) had acceptable growth which confirmed the viability of the strains as shown in Supplementary Figure S1.

It was also observed that Lb. bulgaricus strains S9 and LB6 were fast-growing, especially in the MRS medium, hence their selection for the fermentation study. Formulations two, and four did not yield promising growth results; however, formulation three (Supplementary Figure S2) which included a basal medium with 2g/L of Nucleo-Max (N-Max) in conjunction with various blends of KAT (K) and Carbo-Max (C-Max) yielded exceptional results.

Based on the positive outcome of formulation three, formulation five (5) was developed and consequently chosen as the final optimized recipe for the study. The fifth and final growth media formulation was based on 5% (5g/L) and 10% (10g/L) of the validated nitrogen sources (KAT and C-Max, respectively), and a blend of both KAT and C-Max (KCMax) in a supplemented
basal medium containing 2g/L of Nucleo-Max (N-Max). Thus, the various formulations employed were C-Max 5%, C-Max 10%, KAT 5%, KAT 10%, KCMax (5/5), KCMax (5/10), KCMax (10/5), and KCMax (10/10), all of which were supplemented with a basal medium (2g/L of N-Max). MRS was also used as the control medium in all cases for the fermentation study. The nitrogen source blends KCMax (5/5) represented 5g/L of KAT and 5g/L of C-Max, and KCMax (5/10) was a combination of 5g/L of KAT and 10g/L of C-Max. The blend KCMax (10/5) constituted 10g/L of KAT and 5g/L of C-Max and the final blend, KCMax (10/10) was a combination of 10g/L of KAT and 10g/L of C-Max respectively.

References


Table legends:

Supplementary Table S1:
Probiotic strains used in the preliminary study

Supplementary Table S2:
Total Protein (%) and Nitrogen (%) of validated yeast samples for the study

Supplementary Table S3
Nitrogen sources used for the growth media formulation

Supplementary Table S4:
Composition per liter of deMan Rogosa Sharpe medium (MRS) and the optimized growth medium

Supplementary Table S1

<table>
<thead>
<tr>
<th>No</th>
<th>Product Code</th>
<th>Sample</th>
<th>Source</th>
<th>Bacterial Composition as labeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>S9</td>
<td>Pure Industrial Strain</td>
<td>Bulgaria</td>
<td><em>Lb. bulgaricus</em></td>
</tr>
<tr>
<td>2.</td>
<td>LB6</td>
<td>Pure Industrial Strain</td>
<td>Bulgaria</td>
<td><em>Lb. bulgaricus</em>,</td>
</tr>
<tr>
<td>3.</td>
<td>THT</td>
<td>Pure Industrial Strain</td>
<td>Belgium</td>
<td><em>Lb. bulgaricus</em></td>
</tr>
</tbody>
</table>
Growth Media Formulation | Nitrogen Sources (X-Seed) | Ratio / Blend | Standard
---|---|---|---
1 | Nucleo Max, KAT, Carbo Max, Carbo Peptone, and Peptone | 1% w/v of each nitrogen source | MRS
2 | Nucleo Max, KAT, Carbo Max, Carbo Peptone, and Peptone | 0g/L, 5g/L, 10g/L of each nitrogen source | MRS
3 | Nucleo Max, KAT, Carbo Max, Carbo Peptone, and Peptone | 0g/L, 2.5g/L, 5g/L, 7.5g/L and 10g/L of KAT, Carbo Max, Carbo Peptone and Peptone respectively in a basal medium (2g/L Nucleo Max) | MRS
4 | Nucleo Max, KAT, and Peptone | 1% w/v blend of KAT/Nucleo Max, 1% w/v blend of KAT/Peptone/Nucleo Max and 1% w/v blend of Nucleo Max/Peptone | MRS
Nucleo Max, KAT, and Carbo Max, 5g/L, and 10g/L of blends of KAT, and Carbo Max, respectively in a basal medium (2g/L Nucleo Max)
Supplementary Figure S4a, S4b, S4c, and S4d:
The varying buffering capacities of the C-Max (5% and 10%) medium supplemented with and without L-Arginine.

Supplementary Figure S5a, S5b, S5c, and S5d:
The different buffering capacities of the KAT (5% and 10%) medium supplemented with and without L-Arginine.

Supplementary Figure S6a, S6b, S6c, and S6d:
The varying buffering capacities of the KCMax (5/5) and KCMax (5/10) medium supplemented with and without L-Arginine.

Supplementary Figure S7a, S7b, S7c, and S7d:
The buffering capacities of the KCMAX (10/5) and KCMAX (10/10) medium supplemented with and without L-Arginine.

Supplementary Figure S8a, S8b, and S8c:
The different buffering capacities of the N-Max medium supplemented with and without L-Histidine against the buffering capacity of the control medium (MRS).

Supplementary Figure S9a, S9b, S9c and S9d:
The different buffering capacities of the C-Max (5% and 10%) medium supplemented with and without L-Histidine.

Supplementary Figure S10a, S10b, S10c, and S10d:
The varying buffering capacities of the KAT (5% and 10%) medium supplemented with and without L-Histidine.

Supplementary Figure S11a, S11b, S11c, and S11d:
The different buffering capacities of the KCMAX (5/5) and KCMAX (5/10) medium supplemented with and without L-Histidine.

Supplementary Figure S12a, S12b, S12c, and S12d:
The varying buffering capacities of the KCMAX (10/5) and KCMAX (10/10) medium supplemented with and without L-Histidine.

Supplementary Figure S13:
Calcium concentrations detected in KCMAX (10/10) medium after fermentation with S9 Lb. bulgaricus strain with different treatments of cell wash. Error bars indicate SD for an experiment performed in triplicate.

Supplementary Figure S14:
The bacterial count of S9 *Lb. bulgaricus* strain after calcium supplementation in the fermentation medium KCMAX (10/10) with two different treatments of cell wash. Error bars indicate SD for an experiment performed in duplicate.

Supplementary Figure S1:
Supplementary Figure S2:

Blends/Treatments with 2g/L N-Max (Basal)
Supplementary Figures S3a, S3b and S3c (Arg: Arginine):

(3a) Buffer Capacity of N-Max only

(3b) Buffer Capacity of N-Max-Arg only

(3c) Buffer Capacity of MRS
Supplementary Figures S4a, S4b, S4c and S4d (Arg: Arginine):
Supplementary Figures S5a, S5b, S5c and S5d (Arg: Arginine):
Supplementary Figures S6a, S6b, S6c, and S6d (Arg: Arginine):

(S6a) Buffer Capacity of KCMax (5/5)

(S6b) Buffer Capacity of KCMax (5/5) - Arg

(S6c) Buffer Capacity of KCMax (5/10)

(S6d) Buffer Capacity of KCMax (5/10) - Arg
Supplementary Figures S7a, S7b, S7c, and S7d (Arg: Arginine):

(S7a) Buffer Capacity of KCMax (10/5)

(S7b) Buffer Capacity of KCMax (10/5) - Arg

(S7c) Buffer Capacity of KCMax (10/10)

(S7d) Buffer Capacity of KCMax (10/10) - Arg
Supplementary Figures S8a, S8b, and S8c (His: Histidine):

(S8a) Buffer Capacity of N-Max only

(S8b) Buffer Capacity of N-Max-His only

(S8c) Buffer Capacity of MRS
Supplementary Figures S9a, S9b, S9c, and S9d (His: Histidine):
Supplementary Figures S10a, S10b, S10c, and S10d (His: Histidine):
Supplementary Figures S11a, S11b, S11c, and S11d (His: Histidine):
Supplementary Figures S12a, S12b, S12c, and S12d (His: Histidine):
Supplementary Figure S13:

![Bar chart showing calcium treatments and their effects on cell washes.](chart1.png)

Supplementary Figure S14:

![Bar chart showing calcium treatments and their effects on log CFU/ml.](chart2.png)